# BIOLOGICAL PRODUCTION OF METHANOL BY USING PECTIN METHYLESTERASE (PME)

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## ABSTRACT

Methanol contains only one carbon atom ($CH_3OH$) is the simplest alcohol that also known as wood alcohol. It is a colourless liquid, volatile, flammable and poisonous in properties. It is widely used in several industries such as plastic industry. It also been used as an original feedstock to make polymer. This research study was focus on the production of methanol by using pectin methylesterase (PME). This research study consists of two objectives. The first objective of this research was to produce methanol by using pectin methylesterase (PME). The second objective was to analyse the factors that affecting the methanol production from pectin methylesterase (PME). The raw material, lime peels waste and the substrate, pectin solution were prepared. The extractions of PME from lime peels were done. PME and pectin were mixed for enzymatic reaction process. The experiment for the preliminary study was performed. Preliminary study was performed with five factors which were pH, temperature, concentration of NaCl, agitation and enzymatic reaction time to know the highest production of methanol. Design experiment by using Design Expert software was done after preliminary experiment finished. The analysis to know the production of methanol was performed by using high performance liquid chromatography (HPLC). The result of preliminary experiment by using HPLC showed that the highest concentration produced was at pH 7 and temperature of 70 °C with 5 hours enzymatic reaction time and 3 M of concentration of NaCl. The results of 18 runs sample from Design Expert software were analysed by using Response Surface Methodology (RSM) through Fractional Factorial Design (FFD). The results showed that the range yield of methanol was between 0.3 – 2.2 g/g. The main effect analysis showed that concentration of NaCl had contributed the most in methanol production with percentage contribution of 45.36%. The result of interaction between factors showed that the interaction between factor A (pH) and factor B (temperature) at pH 5 and 70 °C produced the highest methanol with 13.22 % contribution. The value of $R^2$ for the regression analysis was 0.9904. Hence, the methanol was successfully produced from pectin methylesterase (PME) by using lime peels waste.

# ABSTRAK

Metanol mengandungi satu karbon atom (CH3OH) adalah alkohol yang paling mudah yang juga dikenali sebagai alkohol kayu. Ia adalah cecair yang tidak berwarna, tidak menentu, mudah terbakar dan beracun dalam tanah. Ia digunakan secara meluas dalam beberapa industri seperti industri plastik. Ia juga telah digunakan sebagai bahan mentah asal untuk membuat polimer. Kajian penyelidikan ini adalah fokus kepada pengeluaran metanol dengan menggunakan “pectin methylesterase” (PME). Kajian penyelidikan ini terdiri daripada dua objektif. Objektif pertama kajian ini adalah untuk menghasilkan metanol dengan menggunakan (PME). Objektif kedua adalah untuk menganalisis faktor-faktor yang memberi kesan kepada pengeluaran metanol daripada (PME). Bahan mentah iaitu sisa kulit limau dan substrat iaitu cecair pektin telah disediakan. Pengekstrakan PME dari kulit limau telah dilakukan. PME dan pektin bercampur untuk proses tindak balas enzim. Percubaan untuk kajian awal dilakukan. Kajian awal telah dilakukan dengan lima faktor iaitu pH, suhu, kepekatan NaCl, goncangan dan masa tindak balas enzim untuk mengetahui pengeluaran tertinggi metanol. Eksperimen reka bentuk dengan menggunakan perisian “Design Expert” dilakukan selepas percubaan awal selesai. Analisis untuk mengetahui pengeluaran metanol telah dilakukan dengan menggunakan “High Performance Liquid Chromatography” (HPLC). Hasil eksperimen awal dengan menggunakan HPLC menunjukkan bahawa kepekatan tertinggi yang dikeluarkan adalah pada pH 7 dan suhu 70 ° C dengan 5 jam masa tindak balas enzim dan 3 M daripada kepekatan NaCl. Keputusan daripada 18 sampel berlangsung dari perisian “Design Expert” dianalisis dengan menggunakan “Response Surface Methadology” (RSM) melalui pecahan “Full Factorial Design” (FFD). Hasil kajian menunjukkan bahawa hasil julat metanol adalah antara 0.3-2.2 g / g. Analisis kesan utama menunjukkan bahawa kepekatan NaCl telah menyumbang pengeluaran tertinggi metanol dengan sumbangan peratusan 45.36%. Hasil daripada interaksi antara faktor menunjukkan bahawa interaksi antara faktor A (pH) dan faktor B (suhu) pada pH 5 dan 70 ° C menghasilkan metanol tertinggi dengan peratusan sebanyak 13.22%. Nilai $R^2$ untuk analisis regresi adalah 0.9904. Oleh itu, metanol telah berjaya dihasilkan daripada (PME) dengan menggunakan sisa kulit limau.

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| | |
|---|---|
| PME | Pectin methylesterase |
| FFD | Fractional factorial design |
| RSM | Response surface method |
| DM | Degree of methylation |
| HM | High-methoxyl |
| UMP | Universiti Malaysia Pahang |
| $CO_2$ | Carbon dioxide |
| NaCl | Sodium chloride |
| CO | Carbon monoxide |
| $H_2O$ | Water |
| $H_2$ | Hydrogen |
| HPLC | High Performance Liquid Chromatography |
| U.S. | United States |
| RPM | Rotational per minute |
| mL | Millilitre |
| M | Molar |
| w/v | Weight per volume |
| ANOVA | Analysis of Variance |
| NaOH | Sodium hydroxide |
| HCl | Hydrocloride acid |
| | |
| $^{o}C$ | Degree Celcius |
| Rpm | Revolution per minute |
| % | Percentage |

# 1. INTRODUCTION

## *1.1 Motivation and statement of problem*

Methanol was the simplest alcohol that contained only one carbon atom ($CH_3OH$). It was known as wood alcohol. Methanol was a colourless liquid, volatile, flammable and poisonous. It can be produced from different feedstock resources, predominately from natural gas and coal. Methanol was used in several industries such as in plastic industry. It also acts as an original feedstock to make polymer. Methanol also had been proved to be as fuel. When it was blended with gasoline in internal combustion engines, 85% of it was methanol and other 15% was gasoline. It also can be produced as pure methanol. Besides that, methanol also served as a raw material to produced chemical products for example formaldehyde, acetic acid, polymers, paints, adhesives, construction material and synthetic chemicals. (Luzia *et al*., 2011)

Pectin methylesterase (PME) involved in de-esterification of pectin that released methanol and acidic acid. It is a heterogeneous group of enzyme complex that involved in pectin hydrolysis and composed of pectin esterase. The applications of PME were in food industry, textile, wines, pulp and paper industry (Sameer *et al*., 2013). This enzyme catalysed the hydrolysis of the methyl ester group from pectin. It found in plants and also in pathogenic fungi and bacteria (Gayen S., & Ghosh U., 2011). PME had been purified and characterised from several species of citrus fruits such as orange and lemon (John & Tove, 2002). According to John & Tove (2002), citrus fruits were commercially used for juice extraction. Because of the high content in pectin, it also been used in production of methanol.

In this study, PME were be extracted from the citrus fruit that was lime peels waste. Lime peels were being mixed with pectin solution for enzymatic reaction process. Based on the production of methanol, the best factors were being investigated further in order to increase methanol production. The factors that were varied are temperature, pH, fermentation time, concentration of NaCl and agitation.

Fractional analysis by using two levels Fractional Factorial Designs (FFD) was studied in this research. Two level Fractional Factorial Designs (FFD) are popular experimental design and commonly used in engineering analysis (Don, 2013). In addition, FFD allows possible consideration of multi factors and it can determine the most relevant factors from all of the outcomes. Recently, FFD was the analysis that used to investigate the effect of tested independent variables to the response within the investigation range (Khalil *et al*., 2011). Hence, FFD was a technique to determine the influence of several variables on the response and also estimating the overall main factor effects and interaction of different factors (Golshani *et al*., 2013). According to Jawad *et al*., (2013), FFD sign had been used to study the effect of independent variables and the level of selected factors that been chosen for preliminary experiments.

Usually, methanol was produced from synthesis gas where the main gas used is carbon monoxide and hydrogen. In order to produce methanol in typical plant, natural gas feedstock had to convert into a synthesis gas stream that consist of CO, $CO_2$, $H_2O$ and $H_2$. It was usually accomplished by the catalytic reforming of feed gas and stream. Hence, by this synthesis gas method, the methanol produced was highly exothermic and taking place over a catalyst bed at moderate temperature. This condition will generate more energy of electricity and this were increased the capital cost. In this research study, a lime peels were used as the medium to release PME where PME was needed in this research for the production of methanol. They were chosen because it was agricultural or agro-industrial wastes that were abundant, renewable and inexpensive energy source that available in Malaysia. Hence, by PME that extracted from lime peels waste, it provides cost effective and eco-friendly method for the production of methanol on large scale (Patil & Chaundhari, 2010).

### *1.2 Objectives*

The following were the objectives of this research:

- To produce methanol by using pectin methylesterase (PME) from lime peels waste and pectin solution as substrate
- To analyse the factor that affecting the methanol production from pectin methylesterase (PME)

### *1.3 Scope of this research*

This research study was about the biological production of methanol by using pectin methylesterase (PME). Firstly, substrate which is pectin solution was prepared and the PME was extracted from lime peels waste. The fruits were cut into halves and take out the peels. Immediately the peels were treated to extract the pectin enzyme. Then, the preliminary studies were started by varying the factors. Production of methanol based on enzymatic reaction process was analysed by using High Performance Liquid Chromatography (HPLC). Hence, both objectives were completed. In this research study, it was interested to apply optimization tools like Response Surface Methodology (RSM) to improve the production of methanol via Fractional Factorial Design (FFD). FFD can provide clear understanding about the interactions involved between the process variables during the production process (Salleh, H. *et al*., 2011). The screening process and analysing factors by using FFD that was taking into account were temperature, pH, enzymatic reaction time, agitation and concentration of NaCl.

### *1.4 Main contribution of this work*

The following were the contributions of this work. Through this research, it can enrich the knowledge about the production of methanol by using pectin methylesterase (PME) as the raw material. To choose and used pectin methylesterase (PME) as the raw material in the production of methanol, it was the best method since it used agricultural waste as the raw material that was peels from lime where it were low cost and eco-friendly. It also can reduce the exponential increased of greenhouse effect by the polluting action of the industrial and transport sector (Luzia *et al*., 2011).

### *1.5 Organisation of this thesis*

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of the applications and general briefing about the raw material which is pectin methylesterase (PME) and the production. A general description on characteristics of the methanol, as well as the factors that was used in this research study as the parameter to run the experiment in preliminary study was also reported. This chapter also provides a brief discussion of the pectin solution and pectin methylesterase (PME) as the enzyme that were used in this study to produce methanol. A summary of the previous experimental work on the production of methanol by using agricultural waste was also presented. A brief discussion on the screening methods for methanol production and the analysis that was used during the screening was also provided.

Chapter 3 gives an overview of the material and methods that were used in this research. The flow diagram about the process of the experiment was also presented. This chapter also reported about the method of substrate preparation of pectin methylesterase (PME) which is from lime peels. The experimental design by using Response Surface Methodology (RSM) through Fractional Factorial Design (FFD) and the screening factors by using Design Expert software also discussed in this chapter. A brief discussion about the enzymatic reaction process, the preliminary study and also design experiment by five factors was also provided. The review about the analysis of the methanol by using High Performance Liquid Chromatography (HPLC) also presented in this chapter.

Chapter 4 was devoted to an enzymatic reaction process between pectin methylesterase (PME) from the lime peels waste as the raw material and pectin solution as a substrate to produce methanol. In this chapter, result and discussion for the experimental study were presented. There were results for preliminary study and results from Design Expert software that consist of ANOVA and regression analysis, main effect analysis, and interaction between factors. This chapter also discussed about comparison of methanol production with other researcher.

Chapter 5 draws together a summary of the thesis and recommendation which might be derived in this work.

# 2. LITERATURE REVIEW

## *2.1 Overview*

According to Okonko *et al.*, (2009), technology enhancement and human development contributed to the continuous increased in the worldwide energy demand. Bhattacharyya *et al*., (2008) stated there were three categories of energy sources that were fossil fuel, renewable and nuclear energy. The examples of fossil fuels were coal, petroleum and natural gas that were non-renewable energy sources that will be depleted in the next few years. The renewable energy sources included solar, wind, hydroelectric, biomass and geothermal energy whereas nuclear energy was derived from fission and fusion reactions (Gullu & Demirbas, 2001). Fossil fuel sources depletion had increased the need to reduce the consumption of fossil fuels. However, the depletion was not the only current concerned with fossil fuel use but the environmental degradation is. The burning of fossil fuels and the waste products that produced from it had created an imbalance in the atmospheric carbon dioxide ($CO_2$) levels, which had become the major contributor to global warming. While the municipal solid wastes from human and animal activities had also contributed to the environmental degradation. Therefore, it had been suggested that this waste should be recycled or converted into energy (Mastro & Mistretta, 2004).

Biomass was considered to be the renewable energy source with the highest potential to contribute to the energy needs of modern society for both developed and developing economics worldwide (Bridgewater, 2003). According to the 2001 report by the International Energy Agency (EIA), biomass currently contributed about 10.8 % of the world energy supplies including waste while other regenerative energy sources such as hydropower, wind, geothermal and solar were contributed about 3% only (Corradetti & Desideri, 2007). Hall *et al*., (1992) supported that biomass currently supplied the highest proportion of regenerative energy among all regenerative resources with the percentage contribution of biomass as energy sources more than 35% in many developing countries such as tropical Africa.

In 2013, approximately 6.2 billion tons of carbon was emitted into the atmosphere as $CO_2$ and approximately 40% of this was emitted during the production of electricity. A survey from the U.S. Department of Energy revealed that the consumption of electricity increased significantly every year by 44% from 2006 to 2030 (Achmad *et al*., 2011). Leduc *et al*., (2011) stated that by 2050, road transportation was expected to be the largest contributor to greenhouse emission. In Europe, the renewable energy target for 2010 was approximately 5.75% of the transport fuels sold, and this target were likely increased to 10% in 2020. If this trend continues increased, the renewable energy target for the transport fuels sold should reached 27% by 2025.

The worldwide demand of methanol in 2013 was expected to reach 65 million metric tons which driven in a large part by the resurgence of the global housing market and this demand was increased because of the cleaner energy (Methanol Institute). In addition, according to the research from Methanol Institute, the methanol industry will spans the entire world with the worldwide production for Asia, North, South America, Europe, Africa and Middle East was about 33 billion gallons. It also reported that each day more than 100, 000 tons of methanols were used as a chemical feedstock or transportation fuel. They also stated that methanol was a truly global commodity where each day there is more than 80,000 metric tons of methanol will ship from one continent to another. This was due to the high demand of methanol that makes methanol production increased from year to year. In addition, the total cost of methanol production by using biomass was cheaper than the cost of methanol production by natural gas. Thus, biomass processing was the most cost-effective processes that had been developed for the production of methanol from renewable source (Shamsul *et al*., 2014).

Lastly, the rapid growth of chemical technologies and industries that contributed to air and environmental pollution required some limitations to prevent the excessive emission of carbon dioxide into the atmosphere. These problems was leads some researcher to studies about the production of methanol from biomass.

## *2.2 Methanol*

Methanol $CH_3OH$ was a group of alcohol that widely used chemical as a common solvent in organic synthesis. It was the simplest organic liquid hydrogen carrier that acts as a hydrogen storage compound. Methanol also was a liquid transportation fuel that can be produced from fossil or renewable domestic resources. It was an attractive automotive fuel because of its physical and chemical characteristics. In United States, it was the most commonly used as a chemical feedstock, extractant, or solvent for producing methyl tertiary butyl ether (MTBE), an octane-enhancing gasoline additive. It also can be used in net which is 100% pure form as a gasoline substitute, or in gasoline blends. Methanol that produced from biomass had the most potential as a biofuel for power generation because it is distributed form of energy production. Fuel-grade methanol was a clean and efficient alternative fuel that can be used in power industry application for gas turbines. While for the transport sector, methanol acts as a superior to gasoline because it can burns at low temperature. Hence, because of the low volatility, methanol reduced the risk of an explosion or flash fire. The fires can be easily extinguished with water because its characteristics that less flammable than gasoline. This makes methanol more advantageous than hydrogen due to the problems associated with hydrogen storage (Shamsul *et al*., 2014).

Demirbas, A. *et al*., (2011) stated that methanol can be used as one possible replacement for conventional gasoline and diesel fuel. It was a promised renewable fuel that had lower carbon emissions compared to conventional fuel. The used of methanol could also reduce carbon emissions by motor vehicles by up to 81% and up to 32% for the carbon emission if the methanol were used to generate electricity. Methanol was industrially important chemical that acts as a raw material used in many chemical processes (Trop *et al*., 2014). It also mainly used as a feedstock during the production of bulk chemical for example acetic acid and aldehyde (Anita, 2014). According to Hamelinck, (2002), methanol that produced from biomass was a promising carbon neutral fuel. This was because methanol that produced were clean and emitting none of the air pollutant that were $SO_x$, $NO_x$, VOS or dust. Form the research also stated that methanol that were derived from the grown biomass can be greenhouse gas neutral from the overall energy chain. This statement was supported by Kumabe *et al*., (2008) that the fossil fuel emission during fuel processing had prompted the search for renewable sources that emit zero or low pollution. The use of bio-methanol from biomass was more advantageous than fossils products because of its low pollution emission and raw material availability. Hence, biomass was a renewable energy sources that can potentially replace fossil fuels because of the characteristics of this alcohol were identical to those fossil fuels.

The National Renewable Energy Laboratory from U.S. Department of Energy reported that in U.S. industry, it produced approximately 4.7 billion litres of methanol annually. About 38% of these methanols were used in the transportation sector, mostly in the production of MTBE. Most of the methanol produced in the United States today was made from natural gas but methanol also can be produced from other feedstock including coal, biomass and residual oil. Cost-effective, efficient, and environmentally sound processed for produced methanol from biomass make it being pursued by both government and industry research. While in China, since methanol was introduced in 2008, the amount of E85 used in China had included the blending of more than 1 billion US gallons of methanol into fuel (Shamsul *et al*., 2014). This reported showed that methanol was one of the biofuel that had a potential market in the world in future especially when it produced from renewable energy that was biomass.

### *2.3 Previous study on methanol production*

Since a few decades ago, there were many research studies about production of methanol. There had many researchers already doing their research about production of methanol especially by using biogas, natural gas and coal via gasification process also methanol production from $CO_2$. Nowadays there also had many previous studies about production of methanol by using raw material that do not harm environment and can save a lot of cost which was biomass. Trop *et al*., (2014) was done the research study about producing methanol from a mixture of torrefied biomass and coal where this method was the so-called torrecfaction of biomass. Torrecfaction was also known as mild pyrolysis where the process was exposed to $200^{\circ}C$ – $300^{\circ}C$ within anaerobic environment where this method were used high temperature. Hamelinck & Faaij (2006) reported about previous work on methanol by syngas process that includes CO and hydrogen. By this process, syngas is converted into methanol by a catalytic process based on copper oxide, zinc oxide or chromium oxide catalyst. In this process, distillation was used to remove water generated during methanol synthesis. Hence, previous research about production of methanol also was done by using natural gas. This production process can replaced biogas because biogas contains a large share of $CO_2$ compared to natural gas. Production of methanol by natural gas process was used waste anaerobic digestion (Kralj & Kralj, 2009). Bhattacharyya *et al.*, (2008) reported that methanol was also produced from the breakdown of methyl esters or the combination of ether with the methoxyl groups of uranic acid that was produced by the decomposition of pectin-like material plant. Xu *et al.*, (2011) had presented a novel approach for high efficient conversion of the $CO_2$-rich bio-syngas into the CO-rich bio-syngas carried out using biomass char and nickel catalyst, that successfully applied for production of bio methanol by using bio oil. Other than that, there are many researches already produced methanol by using biomass from agricultural waste as a raw material such as sugarcane bagasse, rice straw, rice husk, rice bran, wheat straw and etc. which had high potential in the future.

### *2.4 Pectin as a substrate*

Within numerous enzymes, pectin was one of the best enzymes that can be used in methanol production. Pectin was one of the categories of complex group of associated natural polysaccharides from the primary cell walls and an intracellular region that found in higher plants. It was an important compound for food and pharmaceutical industries in drug delivery systems. The importance of the compound was related to its unique properties and the fact that it was biodegradable. The main raw materials form which commercial pectin was extracted from agricultural by-products, which were citrus peel and apple pomace (Lucyna, L. *et al*., 2013). Wilkins *et al*., (2007) stated that almost half of these citrus fruit was squeezed to juice, and the other remainder including peel, segments membranes and other by-product were considered as citrus waste. These citrus wastes can be dried and used as a raw material for pectin extraction and can be used as the substrate for methanol production (Mamma *et al*., 2008).

According to Wang *et al*., (2002), the dominant component of pectin were galacturonic acid with neutral sugars primarily galactose, arabinose, rhamnose, and xylose. Pectin was used as ingredients in various fields, including medicinal, pharmaceutical, cosmetics and food industries, for its gelling, stiffening and stabilizing properties. It was abundant in fruit and vegetable. Examples of fruits that had high in pectin were citrus, grape, and plum, peach and apple. According to Stephen (1995), the dominant feature of pectin was a linear chain of α-(1, 4)-linked D-galacturonic acid units where varying proportions of the acid group was present as methoxyl (methyl) esters. Generally, galacturonic acid units compose more than 65% of pectin structure where this important structure was the esterification of galacturonic acid residues with production of methanol.

### *2.5 Pectin methylesterase (PME) from extraction of lime peels*

Pectin methylesterase (PME) was the first enzyme that can acting on pectin that was a major component of plant cell wall which it catalyses the de-methylesterification of galacturonic acid units of pectin and generating free carboxyl groups and releasing proton which methanol (Giovane, A. *et al.*, 2004). PME removed methyl groups from pectic component of cell wall during fruit ripening which then can be depolymerised by polygalacturonase and this process will decreased the intercellular adhesively and tissues rigidity (Assis *et al*., 2001). Mustapha, N. *et al*., (2011) stated that de-esterifying enzyme pectin methylesterase (PME) was catalysed the released of methanol and depolymerising enzymes belonging to subclasses that was polygalacturonases. It hydrolysed α-(1, 4) – glycosidic bonds between two non-esterified galacturonic acid residue. These enzymes will act on pectin that acts as a substrate whose degree of esterification was below 55 – 60%. The percentage of carboxyl group that esterified with methanol was called as a degree of methylation (DM). If the DM was more than 50% higher, the pectin was called as high-methoxyl (HM) pectin. But if the DM was less than 50% the pectin was called as low-methoxyl (LM) pectin. Pectin can be de-esterified to produce methanol as it is been abundant in methoxyl side groups. De-esterification of pectin was carried out by a chemical method that was hydrolysation catalysed by using enzyme. The enzyme that been used in de-esterification of pectin was called pectin methylesterase (PME). Pectin methylesterase (PME) was involved in catalysed the de-esterification of pectin. It was produced carboxyl group as well as methanol as products. It also presented in all higher plants but commonly pectin methylesterase (PME) was abundant in citrus fruits (Şİmşek & YemenİcİoǧIu, 2010).

Pectin methylesterase (PME) also catalysed the hydrolysis of the methyl ester group from pectin. The fast and high specific catalysis of pectin methylesterase (PME) make them the economically alternative for hydrolysis of pectin. Pectin methylesterase (PME) was significant to the citrus industry due to the establishment of PME as a causative agent for juice clarification and gelatine of frozen concentrates (Gayen & Ghosh, 2011). According to Nielsen & Christensen (2002), pectin methylesterase (PME) was purified from several fruit that come from citrus family such as lemon, oranges and lime. It was proved that citrus fruits were commercially used in extraction of juices because of the high content of pectin in the fruit. Therefore, detection of a large quantity of pectin in a fruit alone is not in itself enough to qualified that fruit had a higher pectin where pectin were almost exclusively found from citrus peel or apple pomace where both by-product is from juice manufacturing (Thakur *et al*., 1997). Hence, citrus peel contains about 20-30% of pectin that make it suitable to act as enzyme and de-esterified with pectin to produce methanol (May, 1990).

## *2.6 Response surface methodology (RSM)*

Response surface methodology (RSM) was the most popular optimisation method used in recent years. It was developed by Box and collaborators in the 50s century. There were so many works based on the application of RSM in chemical and biochemical process. One of the optimisation techniques in the application of RSM was the selection of independent variables of major effects on the experiment of methanol production through screening studies (Bezerra, M. A. *et. al.,* 2008). The purpose of RSM was to obtain a predicted model and this model can be useful for screening of enzymatic reaction process condition for methanol production. Therefore, in this research study, RSM was used as a screening method in order to increase the yield of methanol production without increased the cost.

RSM was a collection of statistical and mathematical techniques useful for developing, improving and optimising processes in which a response of interest was influenced by several variables and the objective of this method was to screening this response. Hence, it also can be used to define the relationships between the response and the independent variables. RSM had important application in the design, development and formulation of new products, as well as in the improvement of existing product design. It defines the effect of the independent variables, alone or interaction in the process of methanol production. It was possible to separate an optimisation study using RSM into three stages. The first stage is the preliminary work in which the determination of the independent parameters and their levels are carried out. The second stage is the selection of the experimental design and the prediction and verification of the model equation. The last one is the response surface plot and contour plot of the response as a function of the independent parameters and determination of optimum points (Boyaci, H. I. & Bas, D., 2007).

### *2.7 Screening using Fractional Factorial Design (FFD)*

Don (2013) stated that Fractional Factorial Design (FFD) was a popular experimental design method that used for two levels. It was one of the most frequently method that applied fractional design in engineering field. This method made possible to consider multitudinous factors and can identify the most important and relevant factors from the long list of analysis during the enzymatic reaction of methanol production. Khalil *et al*., (2011) stated that recently, FFD was the method in the analysis that used to investigate the effect of tested independent variables to the response within the investigation range during the experimental analysis. Hence, it was also a technique to determine the influenced of several variables on the response and also to estimating the overall main factor effects and interaction of different factors in methanol production (Golshani *et al*., 2013). According to Jawad *et al*., (2013), FFD sign has been used to study the effect of independent variables and the level of selected factors that been chosen for preliminary experiments during methanol production. Xie *et al*., (2003) stated that Factorial Design was a closed-ended system for process optimisation where in this method, level of factors or parameters were independently varied, each factor at two or more levels. This effects that can be attributed to the factors and their interactions were assessed with maximum efficiency for methanol production in factorial design. Moreover, it allow for the estimation of the effects of each factor and interaction. The most commonly used fractional factorials in medium improvement experiments were two factorial designs (denoted by $2^n$ when there are n factors). These designs were the smallest capable of providing detailed information on factor interaction.

## *2.8 Factors used in methanol enzymatic reaction*

During this research study, there must be a few factors that will contribute in order to make sure the methanol can be produced at the end of this research. Hence, in this research study five factors were figured out which these factors can affect the production of methanol. All five factors that had been measured to produce methanol were pH, temperature, agitation, enzymatic reaction time and concentration of NaCl. These factors were the medium that had been used in order to see the successfully of the methanol production in this research study.

### *2.8.1 pH*

pH was a measured of the concentration of hydrogen ions in the solution. The higher the hydrogen ion concentration the lower the pH. Most enzymes function efficiently over a narrow pH ranges. A change in pH above or below this ranges will reduces the rate of enzymatic reaction of that enzyme. Kurita *et al*., (2008) stated that the PME activity increased with the increasing of the pH and the PME activity was higher at neutral pH. The viscosity of the PME was increased gradually from pH 5.8 to pH 7.0 but not after pH 7.6 where after the pH 7.6, the enzyme begins to be denatured. Enzyme denatured was known as the enzyme that start loses their functional shape, particularly the shape of the active site, such that the substrate was no longer fit to it. Hence, according to Carbonell *et al*., (2006), optimum pH where the most favourable pH value that the enzyme was most active for the PME activity for all assayed cultivars was at pH 7.8. But according to Rodriguez-Lopez *et al*., (2013), the highest PME activity was obtained at highest pH that is pH 8.0. As regards of the de-esterification of pectin, high pH would exert a greater influence on the pectin structure and this condition let the pectin become less sensitive to β–elimination that affect the production of methanol (Kurita *et al*., 2008). Spagna *et al*., (2003) reported that optimum pH for the PME extraction was at pH 7. Hence, Amaral *et al*., (2005) and Arotupin *et al*., (2008) was reported in their research that these pH range also affect the PME activity in the production of methanol. In general, PME was found to have an optimum pH ranging from 7.5 to 9.0.

### *2.8.2 Temperature*

Like most chemical reactions, the rate of an enzyme reaction increase as the temperature was raised. In this research study, temperature was one of the important factors that affect the production of methanol. According to Carbonell *et al*., (2006), Atkin & Rouse (1953) had studied about the PME inactivation at different temperatures and found out that PME enzyme was break near 70 $^{\circ}$ C. This means that if the temperature was more than 70 $^{\circ}$C the enzyme were be denatured. Kurita *et al*., (2008), study about the effect of temperature in extraction of pectin. Three different temperatures was used in that method which is 50 $^{\circ}$C, 65 $^{\circ}$C and 80 $^{\circ}$C and found that at temperature 50 $^{\circ}$C was produced high pectin during the extraction. All these statement was supported by Tijskens et al., (1999) and Amaral et al., (2005) in their research that PME had high activity. PME at maximum rate was at the temperature between 50 and 60 $^{\circ}$ C while the enzyme will lose its activity at the temperature higher that 70 $^{\circ}$ C. Therefore, this statement showed that methanol was less produced or cannot be produced if the temperature higher than 70 $^{\circ}$C.

### *2.8.3 Agitation*

In this research study, agitation was one of the factors that need to study because between with agitation and without agitation, there were given a different kind of result towards the PME activity and production of methanol. Although this factor is one of the factor that still new in the research but still had a few researcher that discussed it as one of the important factor that will affect the production of methanol. Garcia-Castello *et al*., (2012) reported in previous research agitation speed at 175 rpm was the best condition of speed for the pectin methylesterase (PME) activity.

### ***2.8.4 Enzymatic reaction time***

Time was one of the factors that also important in this research study in order to produce methanol especially in enzymatic reaction. It was because time will showed at which point of time will be the highest production of enzyme to react with other solution to give the best result of production. According to Rodriguez-Lopez *et al*., (2013), the extraction time for the PME activity to react and produced methanol was significant in the range 20 – 120 minutes. Hence, from the research the finding of the highest PME was reported at time 100 minutes where at this time, the highest methanol was produced. Garcia-Castello *et al*., (2012) in the research study stated and fixed that the extraction time of the PME enzymes between 0.33 - 2 hours and between that ranges, the result that PME activity was highest at 90 minutes where it was a suitable time for the reaction with pectin and then produced a highest methanol.

### ***2.8.5 Concentration of NaCl***

In this research study, concentration of NaCl was used as an extracting solution in order to produced methanol because according to Contreras-Esquivel *et al*., (1999), concentration of NaCl in the extracting solution showed a significant effect on the PME activity measured. This PME when react with pectin solution were produced methanol. The effect of NaCl concentration as extracting solution of PME activity from lime peels showed that the enzyme activity of PME was increased substantially in lime peels when NaCl solutions was used as extractant to extract PME from lime peels. PME from lime peels were extracted with NaCl solutions and in the production of methanol, different concentration between 0.5-3.0 M were used. PME activity was referring as the activity where carboxylic groups were released during the extracting process (Rodriguez-Lopez *et al*., 2013).